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SOME ASPECTS OF THE IMMUNE RESPONSE OF MICE TO THE LIPOPOLYSACCHARIDE ANTIGENS OF GRAM-NEGATIVE BACTERIA

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ABSTRACT

Some aspects of the immune response of mice to the endotoxins of Escherichia coli and Salmonella enteritidis have been investigated. Characterization of the response to both primary and secondary injections of antigen has been done on the basis of both the cellular and humoral aspects of antibody production.

Proper dose levels and intervals between injections have been established for the demonstration of a possible "recall" response to these antigens. The possibility of tolerance induction with concomitant antibody "priming" by a primary injection is discussed.

The rise and decline in antibody-producing cells of the spleen following first and second injections have been shown to follow a pattern closely resembling that demonstrated by circulating antibody. No significant difference was noted between the response to the first and second injection of endotoxin.

Finally, the molecular nature of the response of mice to endotoxins has been characterized. Although variations were noted between the endotoxin preparations, the presence of Immunoglobulin G, (IgG), antibody was detected in the response to all endotoxin preparations tested. Although Immunoglobulin M, (IgM), antibody was always present, a significantly high amount of activity following the second antigenic stimulation was of the IgG type. Heretofore, no involvement of IgG antibody in the immune response to endotoxins has been reported.

INTRODUCTION

The unique chemical and biological properties of the bacterial endotoxins have tended to overshadow their immunologic capabilities as potent antigens. Although Boivin and Mesrobeanu successfully extracted a lipopolysaccharide protein complex from the cell walls of gram-negative bacteria in 1933, a detailed study of the antigenicity of endotoxins was not accomplished until 1955 when Landy et al. demonstrated antibody production in rabbits stimulated with as little as 0.001 ug of a purified Salmonella typhosa preparation. These authors were unable to detect agglutinins in the sera of mice and chickens injected with endotoxin. It should be noted that even in the absence of high agglutinin titers in mouse serum significant protection to challenge with viable organisms has been shown. More recently Rink (1965) observed that mice did produce high-titered agglutinins when injected with selected endotoxic specificities.

Generally speaking the immune response to endotoxin is similar to that seen after a second injection of a protein antigen. This fact along with the recorded evidence for "natural" antibodies in normal mammalian sera has led many investigators to assume that the response to bacterial endotoxins is a secondary response. However, rabbits injected repeatedly with somatic antigens produced very small amounts of Immunoglobulin G, (IgG) antibody (Weidanz, Landy and Jackson, 1964). In preliminary studies Rink (1965) observed that mice produced both IgG and Immunoglobulin M, (IgM) antibodies in response to a single injection of Escherichia coli 0127

endotoxin. Whether the immune response to endotoxins is simply a "typical" secondary response similar to that obtained with protein antigens or is truly a unique response engendered by endotoxins alone remains to be determined.

In the present investigation an attempt has been made to examine this issue by studying both the cellular and humoral aspects of the immune response to selected endotoxins in mice.

REVIEW OF LITERATURE

Generally it has been assumed that mice respond poorly or not at all to injections with the lipopolysaccharide antigens of gram-negative bacteria. In this respect, Doak, Halbert, Smolens and Mudd (1946) observed that mice synthesized much less antibody than did rabbits when injected with Shigella paradysenteriae cells. Cooper, Keller and Hart (1949) were likewise unable to demonstrate a significant amount of agglutinating antibody to Shigella sonnei in mouse serum, although the antiserum was quite effective in protection tests. Landy, Johnson, Webster and Sagin (1955), using Salmonella typhosa endotoxin reported similar negative findings.

Rowley (1955) reported that immunization of mice with Escherichia coli cell wall preparations led to an increased resistance of the animal to infection with antigenically unrelated gram-negative bacteria. The active component of the cell wall preparations was later shown to be endotoxin (Rowley, 1956). Although the exect nature of the increased resistance in these and similar studies is still not well understood, Whitby, Michael, Woods and Landy (1961) reported that endotoxin administration resulted in the release of substances which were active against selected enterobacteriaceae and showed the specificity characteristic of antibodies. Fukui, Chandlee, Klapsogeorge and Berger (1962) and Michael and Massel (1962) obtained data which suggested that antibody was indeed a necessary requirement in the enhancement of host resistance by endotoxin.

Complicating the study of endotoxins has been the exact relationship which exists between the toxic and antigenic portions of the molecule. The crude endotoxin, as extracted from the bacterial cell, has been found to be composed of lipid, protein, and carbohydrate, the amount of each component depending in part upon the method used for extraction of the complex. Of greater interest, however, has been the elucidation of the specific component or components which elicit the observed "endotoxic" effect and the determination of whether this component(s) is synonomous with the antigenic moiety. Such information undoubtedly has a profound bearing on the role of specific antigen-antibody reactions in both the toxic and antigenic manifestations of the endotoxin complex.

Concerning the contribution of the various components of the endotoxin to its physiological and immunological role, Morgan and Partridge (1942) reported that the protein portion of the molecule was necessary for the demonstration of endotoxic properties. However, Webster, Sagin, Landy and Johnson (1955) and Landy et al., (1955) observed that extracts which were essentially protein free were capable of eliciting characteristic endotoxic effects. These findings have been in general agreement with most of the later work in this area (Ribi, Hoyer, Milner, Perrine, Larson and Goode, 1960).

The toxic effects of endotoxins have been attributed to a so called "Lipid A" portion of the molecule by Westphal (1960). This point of view, however, has been the subject of serious controversy stemming from the work of Haskins, Landy, Milner and Ribi (1961), Ribi, Haskins, Milner, Anacker, Ritter, Goode, Trepani and Landy

(1962), Milner, Anacker, Fukushi, Haskins, Landy, Malmgren and Ribi (1963), and Ribi, Anacker, Fukushi, Haskins, Landy and Milner (1964). These investigators have presented evidence suggesting that the endotoxic effect is dependent upon the molecular size of the polysaccharide units and that the role of the lipid is to serve as a cohesive force for the assembly of the active molecule.

Nowotny (1963) demonstrated that the toxicity and pyrogenicity of endotoxin could be diminished by chemical treatment, leaving the serological reactivity, adjuvant effect, and non-specific resistance enhancement factors virtually unchanged. These findings led Nowotny to conclude that the serologically reactive and immunogenic sites were distinct from those which elicited toxic reactions.

Stetson (1964) has stated that all of the major effects of endotoxin could be attributed to antigen-antibody interactions and therefore postulated an immunological basis for the action of endotoxins. In support of this view Nowotny, Radvany and Neale (1965) found that when the toxic "O" antigen of Serratia marcescens was precipitated with specific "O" antibodies the preparation was freed of its toxic properties.

While Kim and Watson (1965) passively transferred endotoxic tolerance by injecting 19S immunoglobulins obtained from tolerant rabbit serum, they found no correlation between the quantity of "O" specific antibody and the ability of the preparation to transfer tolerance. They concluded, therefore, that the "O" antigenic sites were not involved in the toxic manifestations of the endotoxin and the observed tolerance was due to the 19S immunoglobulin which was

specific for distinct toxophore groups on the molecule.

Tate, Douglas, Braude, and Wells (1966) reported that subcutaneous injections of rabbit "O" antisera into mice reduced the lethality of E. coli 0113 endotoxin. A second injection of the antiserum gave added protection and was accompanied by a sharp rise in the "O" antibody titer. However, equally good protection could be afforded with heterologous antiserum. This indicated that the "O" antibody was not necessary for the increased survival rate.

In addition to the numerous physiological and biological activities attributed to endotoxins they have been found to have excellent adjuvant activity. It was recognized early in the study of gram-negative organisms that whole killed cells were capable of enhancing antibody production when administered with other antigens. Johnson, Gaines and Landy (1956) using a number of gram-negative organisms were able to show that the substance active in enhancing the antibody response was a lipopolysaccharide complex. Condie, Staab and Goode (1962) reported that the substance from gram-negative bacteria which was responsible for the adjuvant effect had all the biological properties of endotoxin.

Johnson, et al. (1956) showed that endotoxin was capable of enhancing the antibody response to a number of protein antigens but not to whole cells of gram-negative bacteria. The failure of an adjuvant effect in this respect was attributed to the fact that these cells contain their own endotoxin and further stimulation is not afforded by the external source. It was also concluded by Johnson et al. (1956) that the animal must be susceptible to the toxic action

of the endotoxin before the adjuvant action could be detected. This dependency upon toxicity was in agreement with observations that guinea pigs which are somewhat resistant to the action of endotoxins show no increased antibody response to antigens administered with endotoxin. However, Farthing and Holt (1962) reported that an adjuvant effect could be shown in guinea pigs with the lipopolysaccharides extracted from Bordetella pertussis and E. coli. Furthermore, the Lipid A extracted from these complexes was also an excellent adjuvant without producing the severe stress symptoms seen with endotoxins. This suggested that the stress symptoms produced by endotoxins are not directly related to their adjuvant properties. Merrit and Johnson (1962) also noted an enhanced antibody response in mice, which are quite resistant to endotoxin, when the antigenic stimulus was given in conjunction with endotoxin. With mice the adjuvant effect of endotoxin could be observed if the toxin was given 7 days before or up to 6 days after antigenic stimulation. This is in contrast to a period of 6 hours before antigen administration to 4 days afterward as reported by Johnson et al. (1956).

Observations by Munoz (1961) that B. pertussis vaccine increased the capillary permeability of mice for up to 8 days, led him to speculate that the adjuvant effect of the endotoxins in mice may be due in part to this increase in permeability, (Munoz, 1964) since endotoxins are also capable of eliciting a similar response.

It is apparent then that endotoxins are potentially good adjuvants although the exact mechanism by which they elicit their

adjuvant effect remains obscure. An excellent review of the literature concerning this subject has been written by Munoz (1964).

The complexity of the problems facing investigators in the study of endotoxins can be readily seen from the observations presented above. This probably accounts in part for the poor characterization of the immune response of many animal species to these antigens. It is apparent, however, that basic immunological differences do exist between the lipopolysaccharide antigens and the classical response seen with the protein antigens. In most animals immunization with endotoxins results in an immediate production of antibody, the peak titer being reached in a relatively short period of time. This type of response is not characteristic for an initial stimulus of protein antigens. It is more closely related to the response observed following a second antigenic stimulation. For this reason the immune response to endotoxins has been usually regarded as a secondary type response.

According to Landy et al. (1955) as little as 1 ug of endotoxin injected into rabbits gave a maximum response within three days and significant levels of antibody could be detected for more than three months following injection. However, he obtained no significant response in either chickens or mice immunized with these antigens. Because of these and similar findings, the level of circulating antibody in mice to single and multiple stimulation with endotoxin is not well documented.

Recently, marked differences have been noted in the molecular nature of antibodies produced by an animal following antigenic

stimulation (Fahey, 1962). Uhr (1964), using immunoelectrophoresis, has shown antibody activity to be associated with three classes of gamma globulins, namely IgG, IgA, and IgM. The exact globulin type which will predominate following antigenic stimulation depends upon many factors. Fahey and Lawrence (1962) showed that different animal species responded differently to antigenic challenge with respect to the antibody types which were produced. Likewise, the age of the animal at the time of immunization has been observed to affect the specific globulin type produced (Smith, 1960). Bauer, Mathies and Stavitsky (1963), using phage, Salmonella and protein antigens demonstrated that the nature of the antigen itself will determine in part the molecular nature of the antibody formed. Talmage, Freter and Taliaferro (1956) reported that the intensity of the immunization procedure affected the globulin type which was synthesized. In their studies they used repeated injections of sheep erythrocytes, an antigen which elicits a response more closely paralleling that seen with endotoxins than most of the protein antigens. Finally, Schulman, Hubler and Witebsky (1964) have shown that the antibody type produced may also be affected by the route of immunization.

With many antigens the immune response is characterized by an initial rise in IgM antibody followed by a more rapid appearance of IgG antibody. However, with the lipopolysaccharide antigens the predominant molecular form of the antibody appears to be the IgM globulin. Weidanz, et al. (1964) reported that the repeated immunization of rabbits with purified somatic antigens from gram-negative bacteria did not result in significant levels of IgG antibody. Pike

and Schultz (1964) and Weidanz, et al. (1964) demonstrated that intensive immunization with whole bacterial cells was necessary to produce significant levels of IgG antibody. While Rink (1965) detected IgM and IgG antibodies in mouse serum following immunization with E. coli 0127 endotoxin, no other description of the molecular nature of the antibody formed by mice in response to endotoxin has been reported.

In the last decade several techniques have been developed which makes it possible to characterize the immune response more fully. Nossal (1959) demonstrated antibody production by single cells using a laborious microdroplet technique. This method was quite useful in many respects although it was limited by the small number of cells which could be examined.

A technique which has proven to be most useful in the study of the cellular aspects of the immune response has been that developed by Jerne and Nordin (1963). Sterzl and Mandel (1964) used this procedure to study the inductive phase of antibody formation following injection of mice with sheep erythrocytes. Friedman (1964a) studied the number of antibody competent cells found in various tissues of several strains of mice following immunization with sheep red blood cells. The procedure was further modified by Friedman (1964b) to show the specificity of the plaques formed in the reaction by using two red cell types as indicators.

Landy, Sanderson, Bernstein and Jackson (1964) coated sheep red blood cells with the somatic antigens of S. enteritidis and produced hemolytic plaques with peripheral leucocytes from rabbits

immunized with this antigen. This modification of the original technique made it possible to determine more clearly the response of an animal to immunization with endotoxin, as it was possible to determine the number of cells producing antibody in the various tissues and did not rely on the level of circulating antibody. The method was used quite successfully by Landy, Sanderson, Bernstein and Lerner (1965) to show that the thymus was involved in antibody production to S. enteritidis endotoxin in rabbits. An intensive study of the cellular response of rabbits to endotoxins was done by Sanderson, Jackson and Landy (1965) using this procedure.

Other modifications have afforded a means for the detection of cells producing antibodies of the IgG class, which, because of their low hemolytic efficiency, do not show clearly defined plaques in the usually employed technique.

Sterzl and Riha (1965) and Dresser and Wortis (1965) employing an anti-globulin serum showed an increase in the number of plaques which developed following an incubation of the experimental plates with the antiglobulin preparation. They concluded that the increased plaque count was due to the detection of cells producing 7S antibody which were not evident by the conventional method.

Another useful modification of this procedure was developed by Schwartz and Braun (1965) in which the homologous bacteria were used to detect cells producing antibody to the somatic antigens of the organism. This system is particularly advantageous in that plaques which appear are a result of antibody directed against the antigenic constituents of the bacterial cell, whereas, with the

endotoxin-coated red cells plaques could conceivably arise as a result of natural antibody against the sheep red blood cells.

The plaque formation technique has been used by Friedman (1965) to show the absence of antibody competent cells in the lymphoid tissue of animals made tolerant to sheep red blood cells. Rowley and Fitch (1965) using rats rendered tolerant to sheep erythrocytes reported similar findings using this system.

While antibody plaque formation has been used extensively to study many facets of antibody production, relatively little information has accumulated concerning the immune response to endotoxins. Furthermore, most of the data published in this area has employed rabbits as test animals. The only description of an attempt seen to date to use mice was reported by Michael (1966) who employed lipopolysaccharide antigens extracted from E. coli and selected gram-negative organisms.

The findings presented in this review emphasize the complexity of the problems associated with the study of endotoxins. While much has been learned in the last few years concerning the biological and physical properties of these compounds, their immunological properties have not been well characterized. Relatively few animal species have been used and only relatively few aspects of the immune response have been investigated. A more intensive study of these factors should provide useful information leading to a better understanding of the immunological properties of these compounds.

MATERIALS AND METHODS

A. Experimental Animals

Mice

Male white mice between four and twelve weeks of age were used throughout the experiments. Animals were either of the RML strain, furnished by the Rocky Mountain Laboratory, Hamilton, Montana, or the CD-1 strain purchased from the Charles River Breeding Laboratories, Wilmington, Mass. The mice were housed in metal containers using wood shavings as litter. All animals received Purina "lab chex" and water *ad lib*.

B. Antigens

Endotoxins

A boivin preparation of *Escherichia coli* 0127 lot #466208 was purchased from Difco Laboratories, Detroit, Mich. *Escherichia coli* 0127 (RML) endotoxin, an aqueous phenol toxin extracted from *Escherichia coli* 0127 cells was supplied by Dr. Kelsey C. Milner, Rocky Mountain Laboratory, N.I.A.I.D., N.I.H., Hamilton, Mont.

Escherichia coli 12/13 original endotoxin was an aqueous ether toxin from *E. coli* 0113 cells (H negative and K negative). *E. coli* 15/17 original endotoxin was similarly extracted from *E. coli* 0113 and was almost identical in preparation to the Ec 12/13 original endotoxin.

Salmonella enteritidis (Se 153A) endotoxin was a trichloracetic acid preparation extracted from cells of S. enteritidis S-795.

Salmonella enteritidis (Se Pool #1) toxin was an aqueous ether extract of S. enteritidis S-795. All endotoxin preparations except the E. coli 0127 B toxin was kindly supplied by Dr. Kelsey C. Milner of the Rocky Mountain Laboratory, Hamilton, Mont. All endotoxins were maintained at 4 C in stock solutions of 500 ug/ml in 0.2 per cent formalinized saline and diluted prior to use so that the desired concentration of toxin was contained in 0.25 ml of saline prepared with glass distilled water.

Sheep erythrocytes

Sheep erythrocytes were obtained by bleeding a sheep from the jugular vein and collecting the blood in an equal volume of Alsevers Solution. Prior to use the erythrocytes were packed by centrifugation, washed three times in physiological saline, and finally resuspended in enough saline to give a ten per cent suspension. One-tenth ml of this suspension was administered intravenously via the caudal vein.

Intact bacterial cells

Whole cell antigens were formalin treated E. coli 0127 cells obtained from Dr. K. C. Milner. Bacteria were stored in a stock solution of 500 ug/ml (dry weight) and diluted to yield 50 ug per 0.25 ml immediately prior to use. Injection was made intravenously.

C. Serum

Collecting and processing

Blood samples were collected by cutting the brachial vein of mice under ether anaesthesia. One ml of blood was obtained from each of twelve mice per group with a sterile capillary pipette. The blood was pooled and allowed to clot at room temperature for two hr. The tubes were rimmed and placed in the refrigerator at 4 C overnight. The serum was then separated from the clot by centrifugation at 2,000 rpm for 15 min. The resulting serum was removed with a sterile capillary pipette and immediately frozen until assayed.

D. Assay of Serum

Antigen for bacterial agglutination

Cultures of *S. enteritidis* and *E. coli* 0113 were supplied by Dr. Kelsey Milner. The *E. coli* 0127 culture was originally obtained from the culture collection of the Walter Reed Army Institute of Research through the courtesy of Mr. A. Abrams. All cultures were maintained on Brain Heart Infusion agar slants at 4 C and checked periodically for purity by cultural, biochemical and serological means. Eighteen hr cultures of test bacteria, grown on Brain Heart Infusion agar plates incubated at 37 C, were harvested in approximately 100 ml of formalinized (0.2 per cent) saline. The suspension of bacteria was removed from the surface of the agar and placed in a boiling water bath for a period of one hr. The heat killed cells were then packed by centrifugation, resuspended in formalinized

saline and the stock solution stored at 4 C until used. Immediately prior to use, an aliquot of the stock suspension was adjusted with saline to give a density reading of 25 in a Klett-Summerson photoelectric colorimeter employing a No. 42 filter.

Diluent

Normal rabbit serum (NRS) heated at 56 C for one-half hr and diluted 1:200 in physiological saline was used as the diluent in all cases except where incomplete antibody was assayed.

Test procedure

The serum samples were assayed for antibody activity using the "Microtiter System" manufactured by Cooke Engineering Company, Alexandria, Virginia. Essentially this consisted of mixing 0.025 ml of the antiserum with 0.025 ml of the NRS diluent described above and making serial two-fold dilutions using calibrated wire loops. After titration, 0.025 ml of the test antigen, adjusted to give a reading of 25 Klett Units, was added to each well. Control wells received no antiserum, and each sample was always titrated in triplicate. The plates were then sealed and incubated overnight at room temperature in a humidified chamber. The titer of each sample was recorded as the reciprocal of the highest dilution of the antiserum showing complete agglutination in two of the three replicates.

Occasionally tube agglutination was used to assay serum antibody. This was carried out by adding 0.1 ml of the antiserum to 0.9 ml of NRS diluent and making serial two-fold dilutions with the aid of a 1.0 ml pipette. After dilution 0.5 ml of the antigen

preparation, adjusted to give a Klett reading of 100, was added to each tube. The tubes were then incubated 16 hr at 50 C and placed in the cold for an additional 12 hr. Titers were recorded as the reciprocal of the highest dilution of antiserum showing complete agglutination.

Assay for incomplete antibody

Titration using 15 per cent bovine serum albumin: The method of Gaines, Curry, and Tully (1960) for the detection of incomplete Vi antibody in mice was used. This consisted of titrating the antiserum as described in the tube agglutination test with the exception that 15 per cent bovine serum albumin was used as the diluent.

Use of anti-mouse globulin serum: Serum was also assayed for incomplete antibody by a modification of the Coomb's test. This consisted of incubating the test serum with the antigen preparation for 45 min. then adding one-tenth ml of a rabbit anti-mouse globulin preparation diluted 1:200 prior to use. (Supplied by Dr. J. Munoz). The tubes were then incubated as above and checked for agglutination. Control tubes contained the regular bacterial agglutination mixture diluted to the same concentration as that of the anti-mouse globulin tubes.

E. Solutions and Reagents

Saline

A 0.85 per cent sodium chloride solution was prepared by dissolving 8.5 g of sodium chloride in 500 ml of distilled water and adding enough distilled water to yield a volume of 1.0 liter. The

saline was then distributed in 4 oz prescription bottles and autoclaved at 121 C for 15 min. The sterile solution was stored at room temperature until use.

Phosphate buffered salt solution (PBSA)

Phosphate buffered salt solution was prepared according to the method of Duelbecco and Voght (1954). The salt solutions shown in Table 1 were prepared separately and sterilized at 121 C for 15 min. The solutions were cooled to room temperature, mixed, and enough Albumin Bovine Fraction V, 35 per cent sterile solution (Nutritional Biochemicals Corporation, Cleveland, Ohio) was added to give a 0.4 per cent solution after adjustment of the volume to 1.0 liter. The final volume was distributed into sterile 4 oz prescription bottles and stored at 4 C.

Erythrosin B solution

The erythrosin B stock solution was prepared as described by Phillips and Terryberry (1957). The ingredients were dissolved in 85 ml of distilled water in the order listed in Table 2. The final volume was adjusted to 100 ml and the solution stored at 4 C.

F. Cellular Response

Preparation of cell suspensions

At various time intervals following immunization two mice from each group and two control animals were sacrificed, had their spleens removed aseptically and placed into a petri dish containing a 40 mesh stainless steel screen, and 10 ml of sterile, cold phosphate buffered

Table 1. Phosphate buffered salt solution with bovine serum albumin

Solution A		Solution B	
NaCl	8.00 g	$CaCl_2$	0.10 g
KCl	0.20 g	H_2O	100 ml
Na_2HPO_4	1.15 g		
KH_2PO_4	0.20 g		
H_2O	750 ml		
Solution C		**Solution D**	
$MgCl_2 \cdot 6H_2O$	0.10 g	Bovine Serum Albumin (0.4 per cent) final concentration	
H_2O	100 ml		

Table 2. Composition of erythrosin B solution

NaCl	842 mg
KCl	30 mg
$MgSO_4 \cdot 7H_2O$	25 mg
$NaH_2PO_4 \cdot H_2O$	4 mg
$CaCl_2$	12 mg
Na_2HPO_4	31 mg
Glucose	200 mg
Erythrosin B	400 mg
H_2O	up to 100 ml

salt solution with 0.4 per cent bovine serum albumin. Excess fat was trimmed from the tissue and a cell suspension was prepared by gently scraping the tissue against the wire screen. The resulting cell suspension was then placed in a 16 mm x 150 mm test tube in an ice bath and allowed to stand for 15 minutes in order to separate the cells from the larger tissue fragments. The supernatant was removed and placed into a 12 ml conical centrifuge tube. The cells were packed by centrifugation at 1,000 rpm in a Model PR-2 International Centrifuge (International Equipment Company, Boston, Massachusetts) employing a #269 swinging bucket rotor at 4 C for 10 min. The cells were washed two additional times in an identical manner using PBSA. After the final wash a volume of PBSA equal to the volume of packed cells was added to the tube and the cells were resuspended by gentle agitation. This concentrated suspension was placed in an ice bath until counted.

Viable cell counts

The number of viable cells in the spleen preparation was determined by the method of Phillips and Terryberry (1957). A 0.01 ml aliquot of the cell suspension was mixed with 1.99 ml of PBSA and 0.4 ml erythrosin B solution. A drop of this mixture was then placed on a Spencer Bright-line hemocytometer (American Optical Company, Buffalo, New York) and the number of nucleated non-stained cells in 80 small squares was counted. The number of viable cells per ml of the original suspension was then determined by the formula:

Unstained Cell Count X 1.2 x 10^7 = Viable Cells/ml Suspension

The volume of the suspension was then adjusted with PBSA so that 0.1 ml of the suspension contained 1 x 10^6 viable spleen cells.

Antibody plaque method

The procedure developed by Jerne and Nordin (1963) and modified by Schwartz and Braun (1965) was used to detect cells in the spleen of mice producing antibody to endotoxin. A base layer of 20 ml of Brain Heart Infusion agar was poured into sterile, disposable, 100 mm x 15 mm plastic petri dishes and allowed to solidify. One-tenth ml of the respective bacterial suspension, adjusted to give a density reading of 125 on a Klett-Summerson photoelectric colorimeter, was added to a Wasserman tube containing 2.5 ml of 0.4 per cent melted agarose (Bausch and Lomb) in PBSA maintained at 50 C. One-tenth ml of the spleen cell suspension containing 1 x 10^6 viable nucleated cells was rapidly mixed with the seeded agarose and immediately poured onto the top of the base layer of Brain Heart Infusion agar. The plates were allowed to solidify and then placed in a 37 C incubator for one hr to allow for diffusion of antibody from the antibody competent cells. Complement was then added to all plates in the form of 5 ml of pre-colostral calf serum. The plates were placed in a refrigerator at 4 C for one hr to allow for diffusion of the complement in the absence of bacterial growth. The plates were then incubated an additional hour at 37 C. The complement was removed and the surface of the plates was washed twice with cold PBSA. All plates were then incubated at 37 C until the bacterial lawn became

readily visible. Antibody producing cells were detected as bacteriolytic plaques in the bacterial lawn. The plaques were readily visible and were counted macroscopically using a Quebec colony counter (American Optical Company, Buffalo, New York). The number of competent cells was expressed as the number of antibody producing cells per million nucleated spleen cells. Counts were always made on duplicate plates.

IgG antibody by agar plaque method

The method of Sterzl and Riha (1965) was followed essentially as described for the detection of cells producing IgG antibody during the response of mice to a second injection of endotoxin. The method was exactly as described above for determination of antibody producing cells, except that prior to the addition of complement 5 ml of rabbit anti-mouse globulin serum diluted 1:200 was added and the plates placed at 4 C for one hr. The surface of the agar was then washed twice with PBSA, the complement added, and the plates incubated as usual. Plaque counts on these plates were compared to plates lacking anti-globulin serum, and the difference in plaque counts was assumed to be due to spleen cells producing IgG antibody.

G. Passively Administered Antibody

Disappearance of passively administered antibody

The rate of disappearance of passively transferred antibody was determined by the injection of 0.3 ml of the antiserum intravenously

into the recipients. The agglutinating activity after one, three, and five days was then determined. The passively transferred antiserum was prepared in animals from the same colony and of the same age group as the recipients. Antibody was stimulated in the donor mice by either one or two injections of the endotoxin as outlined in the text.

Immune suppression studies

Attempts to block the immune response to endotoxin were carried out according to the method of Rowley and Fitch (1964). Twenty-five mice were divided into five groups of five animals each. Two groups were given intravenous injections of 0.3 ml of a high titered antiserum. Twenty-four hr later one of these groups was given an intravenous injection of 50 ug of the same endotoxin preparation used to stimulate the production of antibody in the donor animal. The second group received no endotoxin and served as a control to show that any antibody formation was a result of antigenic stimulation and not passively transferred antibody. A third and fourth group were injected with 0.3 ml of normal mouse serum and a mouse anti-sheep erythrocyte serum respectively. The fifth group received no serum transfer. Similarly, the last three groups received 50 ug of the endotoxin twenty-four hr following injection of the respective sera. All animals were bled and the antibody levels of the sera were determined on days five and seven following antigenic stimulation as this corresponded to the time intervals when circulating antibody should have been at its peak.

In cases where it was desirable to determine the effect of passively transferred antibody on the response of mice to a second injection of the antigen, the receipient mice were injected with 50 ug of the endotoxin two weeks previous to the transfer of the antiserum.

H. Immunoglobulin Types

Sucrose density ultracentrifugation

Sucrose density centrifugation was performed on serum samples according to the method of Edelman, Kunkel, and Franklin (1958). The samples to be assayed were diluted 1:2 in 0.05 M phosphate buffer and centrifuged for 20 minutes at 1,500 rpm to clarify the serum. Sucrose gradients (10-40 per cent) were prepared using a "Densigrad" gradient maker (Buchler Instruments, Inc., Fort Lee, N. J.). This was accomplished by mixing 2.2 ml of 40 per cent sucrose with 2.0 ml of the 10 per cent sucrose solution and layering the mixture into a 5 ml cellulose acetate tube (Spinco #5050). One-half ml of the diluted serum was carefully layered onto the sucrose gradient and the tube placed into a previously cooled swinging bucket rotor (Spinco SW39L) and centrifuged for 16 hr at 35,000 rpm in a Beckman Model L preparative ultracentrifuge.

At the end of the centrifugation the tubes were removed from the rotor and mounted in a piercing unit. The bottom of each tube was pierced and 20 drops of the gradient were collected into each of 20 Wasserman tubes.

Each of the fractions was mixed with 1.5 ml of saline and immediately transferred to a Beckman DB spectrophotometer cuvette, and the protein concentration of the sample was determined by measuring the optical density at 280 mu. The fractions were then assayed by the microtiter system previously described to determine the agglutinating activity of each fraction.

Sensitivity to 2-mercaptoethanol

Sensitivity of the serum samples and fractions was determined by the method of Sahiar and Schwartz (1965). Each serum sample and fraction from the sucrose gradient to be analyzed was mixed with an equal volume of 0.2 M, 2-mercaptoethanol. The mixture was incubated at 37 C for one hr and then assayed for antibody activity by bacterial agglutination.

RESULTS AND DISCUSSION

The ability of an animal to respond to antigenic stimulation is dependent to some extent upon the age of the animal at time of injection. This immunological competency is, in part, a reflection of the age at which various lymphoid tissues of the animal matures. Therefore, the first series of experiments were designed to determine the age at which mice responded maximally to a single intravenous injection of endotoxin. For this purpose a 5 ug dose of either E. coli 0113 (Ec 12/13 original) endotoxin or S. enteritidis (Se 153A) endotoxin was employed to immunize mice of the RML strain 6, 9, and 12 weeks of age, as shown in Table 3.

Significant agglutinin titers were not detected in the sera of these mice. While this finding is in agreement with the observations of Landy et al., it differs from the previous observations by Rink (1965) who, using CD-1 mice, suggested that this strain of animal was capable of responding to injections with E. coli 0127 endotoxin showing significant titers to as little as 0.005 ug of the preparation. Although titers were consistently higher with this preparation, a significant response was also noted with S. bulawayo and S. typhosa 0901 endotoxin.

A number of factors are known to have an effect on the ability of an animal to produce antibody in response to antigenic stimulation. The strain of mice used in these studies could have been incapable of responding to the antigen, as all strains of mice would not be expected to respond to the same degree to a given antigen (Gaines, et al. 1960). However, it is also possible that the antigenic challenge was not within the proper dose range required

Table 3. Failure of RML mice to synthesize antibody following stimulation with 5 ug of endotoxin.

Days after Immunization	Antibody Titer*					
	Ec 12/13 toxin			Se 153A toxin		
	6 Wk	9 Wk	12 Wk	6 Wk	9 Wk	12 Wk
3	0**	0	0	0	0	0
5	10	10	0	0	0	0
7	10	10	10	10	10	10
10	20	10	0	10	10	10
14	10	0	10	10	10	10
21	10	0	10	20	20	10
28	0	0	0	10	0	10
35	0	0	0	10	0	0

*Antibody titers are expressed as reciprocals of the highest dilutions of antisera showing complete agglutination.

**0 designates a titer of less than 10.

to stimulate antibody production. While rabbits respond readily to very low doses of endotoxins and are extremely sensitive to the toxic manifestations of most of these preparations, mice are quite resistant to the toxic action of endotoxins, and attempts to demonstrate high agglutinin titers have generally been unsuccessful regardless of the dosage level employed. Furthermore, the polysaccharide antigens of Diplococcus pneumoniae are capable of producing immunological paralysis in mice if an excess of the antigen is injected. Similar findings have not been reported with the lipopolysaccharide antigens of gram-negative bacteria.

Mice which have been selected on the basis of antibody production following immunization with B. pertussis were next obtained from a colony maintained by Dr. J. Munoz, Rocky Mountain Laboratory, Hamilton, Montana. These will be referred to as RMLM mice in the text. The ability of these mice to produce antibody in response to endotoxin stimulations was compared with that of the RML mice used in the preceding experiment.

It is apparent from the results of Table 4 that both groups of mice responded in a similar manner. The peak titers were reached by the fourth day followed by a progressive decline in antibody levels. The best response was noted with the S. enteritidis (Se Pool 1) endotoxin. This preparation elicited higher titers for longer durations than either of the other two preparations. This is possibly a reflection of the purity of the preparation as both Se 153A and Se Pool 1 was extracted from the same organism, Se 153A being a trichloroacetic acid preparation, and Se Pool 1 being an aqueous

Table 4. Comparative antigenicity of three different endotoxin preparations in RML and RMLM mice.

Antigen	Dose	Antibody Titer							
		RML mice				RMLM mice			
		Days after immunization							
		0	4	8	12	0	4	8	12
Ec 12/13	50 ug	0*	0	0	0	0	0	0	0
	5 ug	0	0	0	0	0	0	0	0
	0.5 ug	0	0	0	0	0	0	0	0
Se 153A	50 ug	0	160	80	40	0	160	80	80
	5 ug	0	40	40	0	0	40	20	0
	0.5 ug	0	40	0	0	0	20	0	0
Se Pool 1	50 ug	**	**	**	**	0	320	320	320
	5 ug	**	**	**	**	0	320	320	40
	0.5 ug	**	**	**	**	0	160	80	0

*0 designates a titer of less than 20.
**Not tested.

ether toxin. It is also possible that the preparations may differ in their antigenicity as well.

From these data it can readily be seen that the dose of endotoxin employed is a critical factor governing the ability of the mouse to respond to these antigens. While some antibody was produced to the lower doses of Se 153A endotoxin, significant amounts of antibody were produced only at the 50 ug dosage level. In all cases the duration of the response seemed to be dependent upon the amount of endotoxin injected.

Of interest in this experiment was the fact that neither strain of mice responded to E. coli 0113 endotoxin. The failure of the animals to make antibody to this antigen is difficult to explain. Reports by Gaines, et al. (1960) that mice made incomplete antibody to the Vi antigen of S. typhosa suggested that the antibody synthesized to the Ec 12/13 original endotoxin may be of this nature.

To determine whether or not incomplete antibody was involed in this response, the method of Gaines, et al. (1960) was followed. The antisera were titrated using 15 per cent bovine serum albumin as the diluent. As shown in Table 5 the presence of incomplete antibody could not be demonstrated by this procedure. Furthermore, titration of the samples by the procedure described by Kabat (1961) using a modification of the Coomb's test for detection of incomplete antibody did not result in the detection of agglutinins.

The effect of Freund's complete adjuvant on the response of mice to these antigens was then tested. The antigen was mixed with the adjuvant and injected subcutaneously into the mice. In addition

Table 5. The absence of incomplete antibody in the sera of mice immunized with Ec 12/13 original endotoxin.

Dose	Agglutinin Titer			
	Days after immunization			
	0	4	8	12
50 ug	0*	0	40	0
5 ug	0	0	0	0
0.5 ug	0	0	0	0

*0 designates a titer of less than 20

to the Ec 12/13 original endotoxin another toxin from E. coli 0113, (Ec 15/17 original), was also employed. As can be readily seen from the results in Table 6 the adjuvant did not affect the ability of the animals to respond to the Ec 12/13 original toxin. The other preparation from E. coli 0113, Ec 15/17 original, however, elicited a significant response in the mice whether incorporated into the adjuvant or not. As expected, Freund's adjuvant did not enhance the antibody response of mice to immunization with endotoxin.

Having established the proper dose range for immunization, it was then of interest to determine what effect, if any, a second stimulation would have on the antibody response. Although the literature is not well documented on experiments of this nature, most attempts to produce higher levels of circulating antibody to repeated injections of endotoxins have been at best only partially successful. Table 7 summarizes the response of six-week old mice following a second injection of 50 ug of endotoxins, twenty-seven days after an initial dose of 50 ug. Both injections were given intravenously.

These data demonstrated that the RML strain of mice was capable of responding to a second injection of endotoxin with extremely high agglutinin titers. Although this increased agglutinin response resembled, in many ways, a typical secondary response to protein antigens, certain basic differences were apparent. One characteristic of the classical anamnestic response to most protein antigens is a rapid appearance of high levels of circulating antibody, the peak response being reached in a much shorter period of

Table 6. The effect of Freund's complete adjuvant on the immune respones of mice stimulated with a single subcutaneous injection of 50 ug of endotoxin.

Treatments	Antibody Titer				
	Days after immunization				
	0	3	7	12	19
Ec 12/13 orig. in Freund's	0*	0	0	0	0
Ec 12/13 orig. in saline	0	0	0	0	0
Se Pool 1 in Freund's	0	160	640	80	160
Se Pool 1 in saline	0	320	320	160	160
Ec 15/17 orig. in Freund's	0	20	160	160	80
Ec 15/17 orig. in saline	0	80	160	160	80
Freund's alone	0	0	0	0	0

*0 designates a titer of less 20.

Table 7. Agglutinin response in mice following a second injection of endotoxin twenty-eight days after primary stimulation.

Antigen	Antibody Titer												
	Days after stimulation												
	0	1	4	7	14	20	26	28	1	4	7	14	21
Ec 12/13 orig.	0	0	0	0	20	20	0	**	20	5120	5120	1280	1280
Se 153A	0	20	160	80	160	80	40	**	160	1280	2560	1280	640
Se Pool 1	0	20	160	640	320	160	20	**	80	2560	5120	2560	640

*0 designates a titer of less than 20.
**second injection given on day 28.

time than that following a primary stimulation, and remaining at this elevated level for a long period of time. In these experiments, however, the time of appearance of antibody coincided very closely with that noted following the first injection. Although the titers remained high for a longer period of time following the second stimulation the rate of decline in the amount of circulating antibody seemed proportional to that noted in the initial response. The persistence of antibody over the longer period of time could be the result of the higher levels of antibody which would take longer to disappear by the normal immunoglobulin decay route. If the persistence of the antibody was a result of continued active synthesis, as would be expected in a true secondary response, the antibody level should remain at peak titer for a longer period of time.

It should be noted, however, that with certain lipopolysaccharide antigens the initial injection of the antigen results in circulating antibody within a few days, the peak response being noted usually between the third and seventh day following immunization. This has been considered to result from previous stimulation of the animal by gram-negative organisms of the intestinal flora (Miler, Sterzl, Kosta, and Lanc, 1964). Landy and Weidanz (1964) showed the presence of naturally occurring antibodies to a wide variety of gram-negative organisms in several different species of animals. Consequently, the injection of endotoxin from one of these organisms results in a secondary type response in these animals. With many animal species, notably the rabbit, the criteria of a true secondary response seems to be fully met. However, the exceedingly

low titers noted in mice following a primary stimulation with these antigens, indicates that the response of these animals may be of a different nature than that observed in the rabbit. If the first injection resulted in a true secondary response, then one would expect very high titers following the first antigenic stimulation. Furthermore, Diener and Nossal (1966), using the toad, Bufo marinus, in which immunological memory does not appear to exist, have gained information which suggests that the immune response described above represents a true primary response and may not be the result of prior antigenic stimulation. In view of the many characteristics of this class of antigens it is conceivable that this early appearance of antibody is merely characteristic of these compounds and does indeed represent a true primary response.

It is also apparent that the appearance of detectable antibody after the first stimulation is not a prerequisite for the enhanced antibody production to the second injection. Note that equally high titers were obtained with the E. coli 0113 endotoxin to which no response was obtained on the first injection. The possibility that a dose of 50 ug of the E. coli 0113 toxin was not sufficient to elicit an immune response in mice was investigated. One group of mice was injected with 100 ug of endotoxin and a second group was given two 50 ug injections seven days apart. The group receiving only one injection did not show a detectable agglutinin response. The animals receiving the two injections, however, responded with high antibody titers when tested five days later.

Demonstration of immunological memory to an antigen is dependent upon several factors. The interval between injections as well as the antigenic dose employed affect the ability of the animal to display a secondary response. It was therefore desirable to determine what effect varying these factors would have upon the response noted in the previous experiments to a second antigenic challenge. Table 8 shows the effect of giving the second injection 14 and 35 days following the initial antigenic stimulation with S. enteritidis endotoxin (Se Pool 1).

An interesting development in this experiment was the fact that the initial injection of the antigen did not result in the production of antibody, a phenomenon which had originally been noted only with Ec 12/13 endotoxin from E. coli 0113. However, the second stimulation again resulted in high antibody levels. As can be seen from these results the best response was noted when the second dose of antigen was given 35 days following the primary stimulus. Although the rise and decline in titers followed a similar course, whether the second dose was given 14 or 35 days after the first injection, a delay in the time of the second stimulation resulted in

Table 8. Effect of the time interval between injections on the immune response to a second injection of endotoxin.

Days after antigen	Antibody Titer		
	No second injection	Second injection on day 14	Second injection on day 35
0	0*	0	0
1	0	8	16
2	0	0	0
3	0	256	256
4	0	256	4096
5	0	2048	8192
7	0	4096	8192
14	0	1024	8192
21	0	1024	4096

*0 designates a titer of less than 4.

following second injection on day 14. However, if the animals synthesized antibody to the primary stimulus, it was of such nature as to remain undetectable by the assay system employed, or it was present in such low amounts as to be below the limits of the sensitivity of the bacterial agglutination test. Such low amounts of antibody, however, would not be expected to alter the second response by complexing with the antigen.

Several possibilities exist as a possible explanation for the failure of antibody synthesis after the first antigen injection. First of all, it may be a characteristic of the particular antigenic preparations being used. This seems highly unlikely, however, since the same preparations were used in this experiment as was used in those described in Table 7 where a significant response was noted to the first dose of Se Pool 1 antigen.

Another possible explanation is that the response noted is a reflection of changes in either the genetic constitution of the animals or in the intestinal flora of the mice. The role of genetic factors involving the immune response of these animals is hard to assess, especially since these animals were not obtained from an inbred colony. However, the rapid transition from responsiveness to complete unresponsiveness in all animals tested, strongly argues against this point of view. On the other hand, it is possible that the ability of the mice to respond to these antigens is dependent upon prior exposure to these or antigenically related organisms. Still, it is unlikely that these mice had not received prior stimulation as they were conventionally reared animals and the intestinal

tract of such animals would undoubtedly contain antigenically related organisms, thereby providing a constant stimulus to the animal. While agglutination titers was not demonstrated in these animals prior to injection of the antigen, the use of more sensitive methods such as the bactericidal test was not employed. Such experiments may have shown the presence of antibody which could not be detected by bacterial agglutination. A change in the intestinal flora of these mice is not, in itself, a wholly satisfactory answer to the variation in the responsiveness to the first injection of these antigens.

Recent observations by Nossal and Austin (1966) are pertinent to the issue of non-responsiveness to a primary injection. Using flagellin they were able to induce a state of tolerance to the antigen, while at the same time priming the animal for a secondary response. While no antibody was demonstrated following the primary injection of the antigen, stimulation with a second dose of the antigen at a later time resulted in the production of higher levels of antibody which reached peak titers at an earlier time than observed after a primary stimulation with antibody producing doses of the antigen. The induction of this tolerance and concomitant "priming" was a dose-dependent phenomenon and was interpreted by Nossal and Austin to lend support to their theory that antigen which becomes fixed onto reticular cell processes will stimulate antibody production while antigen taken directly into the cell will induce tolerance.

While tolerance has not been shown to the polysaccharide antigens of gram-negative bacteria, it is tempting to postulate a

system such as that described by Nossal and Austin, on the basis of the similarities which exist between the responses noted in the two systems. Indeed, the results previously reported for the endotoxins in mice show a striking resemblance to the response described following injection with flagellin. One difference is that with the endotoxins, the titer does not seem to rise appreciably following the secondary stimulation. This, however, may be due to differences in the properties of the antigens employed. Further investigation of this facet to the endotoxin response in mice is warranted.

Regardless of the mechanism whereby these mice are rendered unresponsive to injection with endotoxins, the data presented does confirm that demonstration of high agglutination titers in the mouse following immunization with endotoxins is dependent upon a prior exposure of the animal to the antigen. Indeed, in none of the experiments using this strain of mice were titers noted which were comparable in magnitude to those obtained after a second injection of the antigen.

It next became desirable to ascertain whether or not this penomenon was limited to the high level of toxin used in the previous experiments. If a true immunological memory was operable in these mice, one would expect lower doses of the toxin to give increased antibody titers on a second stimulation. For these studies a trichloroacetic acid toxin from E. coli 0127 was used in conjunction with the S. enteritidis preparation (Se Pool 1). The E. coli 0127 endotoxin was a commercial preparation obtained from Difco Laboratories and had the characteristic of eliciting high antibody

titers following a primary stimulus. The results of varying both the first and second doses are shown in Table 9.

This confirms the observations noted in Table 6. The antigenic dose employed determines the magnitude of the immune response to the second injection. The ability of mice to respond to the second antigenic stimulus decreases proportionately with the amount of antigen employed. It should be noted also that the duration of the response is significantly shortened by decreasing the antigen dose given on the second injection. This would indicate that constant stimulation of the antibody synthesizing mechanism, possibly by persistence of the antigen in the system, may be a necessary requirement for the enhancement of the titers. The dose dependency of this response is dramatically apparent from the results obtained when only 0.05 ug of toxin was given at both injections. In this case a significant agglutinin titer could be demonstrated only at day 2 following the second injection.

With E. coli 0127 endotoxin a similar response was noted. The effect of the dose level on the second response is not as apparent, however, with this toxin, possibly because it is capable of eliciting a very good response to a first injection. These data illustrate the fact that the demonstration of this "recall" phenomenon is dependent upon the selection of the proper dose levels in both the first and second antigenic challenges.

Since single or multiple doses of E. coli 0127 endotoxin are capable of stimulating agglutinin titers, it constitutes a potentially good antigen to be utilized for studying the differences

Table 9. The effect of the dose of endotoxin on the immune response of primed mice.

Antigen	Dose 1st inj.	Dose 2nd inj.	Antibody Titer: Days after stimulation 0	1	2	3	4	5	7	14	21
Se Pool #1	50 ug	0	0*	0	0	0	0	0	4	8	8
	50 ug	50 ug	0	0	8	1024	8192	8192	8192	4096	4096
	50 ug	0.5 ug	0	8	16	512	512	2048	512	512	256
	50 ug	0.005 ug	0	8	16	256	256	128	0	0	0
	5 ug	0	0	0	0	0	0	0	8	8	4
	5 ug	5 ug	0	8	8	1024	2048	512	256	256	128
	0.05 ug	0	0	0	0	8	0	0	4	4	4
	0.05 ug	0.05 ug	0	4	256	0	4	0	0	0	0
E. coli 0127	50 ug	50 ug	4	8	**	256	**	1024	1024	512	128
	50 ug	50 ug	4	256	1024	2048	8192	2048	2048	2048	1024
	50 ug	0.5 ug	4	1024	2048	4096	4096	512	512	512	512
	50 ug	0.005 ug	4	2048	1024	**	2048	512	512	256	512

*0 designates a titer of less than 4.
**Lost samples.

between the initial and secondary responses. Since certain similarities were noted between the primary and secondary response to endotoxin when antibody was produced in both cases, the next efforts were directed toward a cellular characterization of these responses. Spleens were removed from normal mice and mice immunized with 50 ug of E. coli 0127 endotoxin, and Jerne plates were prepared as previously described. Figure 1 depicts the response observed following a single injection of endotoxin. The results are expressed as the number of antibody producing cells per one million viable nucleated spleen cells. This probably represents a more accurate account of the response than attempts to determine the total number of cells present per spleen since the recovery of all the cells from a given tissue is rather difficult to achieve.

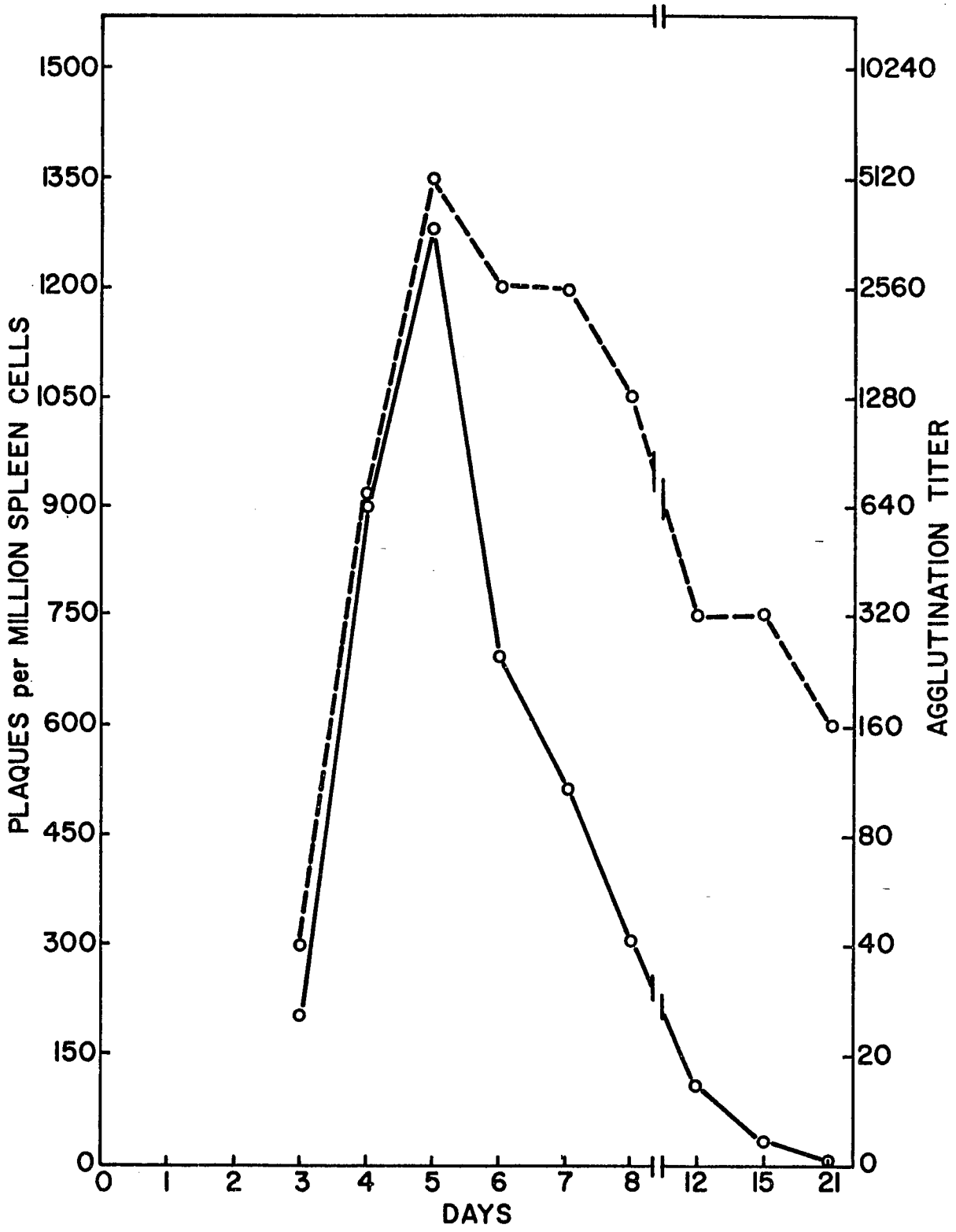
It is recognized that tissues other than the spleen undoubtedly contribute significant amounts of antibody to this response, especially in the latter stages of antibody synthesis. However, no attempts were made to assess the contribution from these other sources as previous experiments had shown the spleen to be the major site of antibody synthesis following the intravenous injection of endotoxin.

A significant aspect of the response to this particular endotoxin is the fact that relatively high natural serum agglutinins are nearly always present as compared to their absence in most of the other preparations used in these studies. Consequently, it was always possible to demonstrate antibody-producing cells in the spleens of normal mice. The usual range was between 5 and 20

Figure 1. Antibody titer and plaque-forming cells per million spleen cells following the injection of 50 ug of *E. coli* 0127 endotoxin.

O——O Plaque count

O----O Agglutinin titer


PLAQUES per MILLION SPLEEN CELLS
1500
1350
1200
1050
900
750
600
450
300
150
0
AGGLUTINATION TITER
10240
5120
2560
1280
640
320
160
80
40
20
0
0 1 2 3 4 5 6 7 8 12 15 21
DAYS

antibody-forming cells per million spleen cells. The magnitude of the antibody titer was of the order of 10 to 20.

The first significant rise in both the antibody titer and the number of plaque-forming cells occurred on day 3 following antigenic stimulation when the antibody producing cells numbered 207. The agglutinin titer at this time was 40. Antibody-producing cells and the agglutinin titers closely paralleled each other while rising to a peak on day 5 when a titer of 5120 and a plaque count of 1305 was attained. Following the peak response there was a very rapid reduction in the number of plaque-forming cells to 690 on day 6 and 117 by day 12. Only 7 competent cells per million spleen cells remained at the end of 21 days. Although the reduction coincided with the fall in antibody-synthesizing cells, the decline from the peak agglutinin titer followed a slower course. This is not surprising, however, since the rate of immunoglobulin decay would not be expected to parallel the transition from cellular competence to incompetence. It is interesting, however, that an antibody titer of 160 still remained 21 days after stimulation, at a time when the number of plaque-forming cells had fallen to a level well within the range of unstimulated animals. This suggests the presence of an immunoglobulin type capable of remaining in circulation for an extended period of time or else a shift in the site of antibody synthesis, as this low number of cells would not be expected to maintain antibody levels of this degree. The immunoglobulin types produced by mice in response to these antigens will be considered in detail in a future section.

These results are in general agreement with those reported by Michael (1966); however, both the cellular and humoral responses reported here are of greater magnitude and of longer duration than those observed by Michael. It is possible that these differences are a reflection of the strain of mice used.

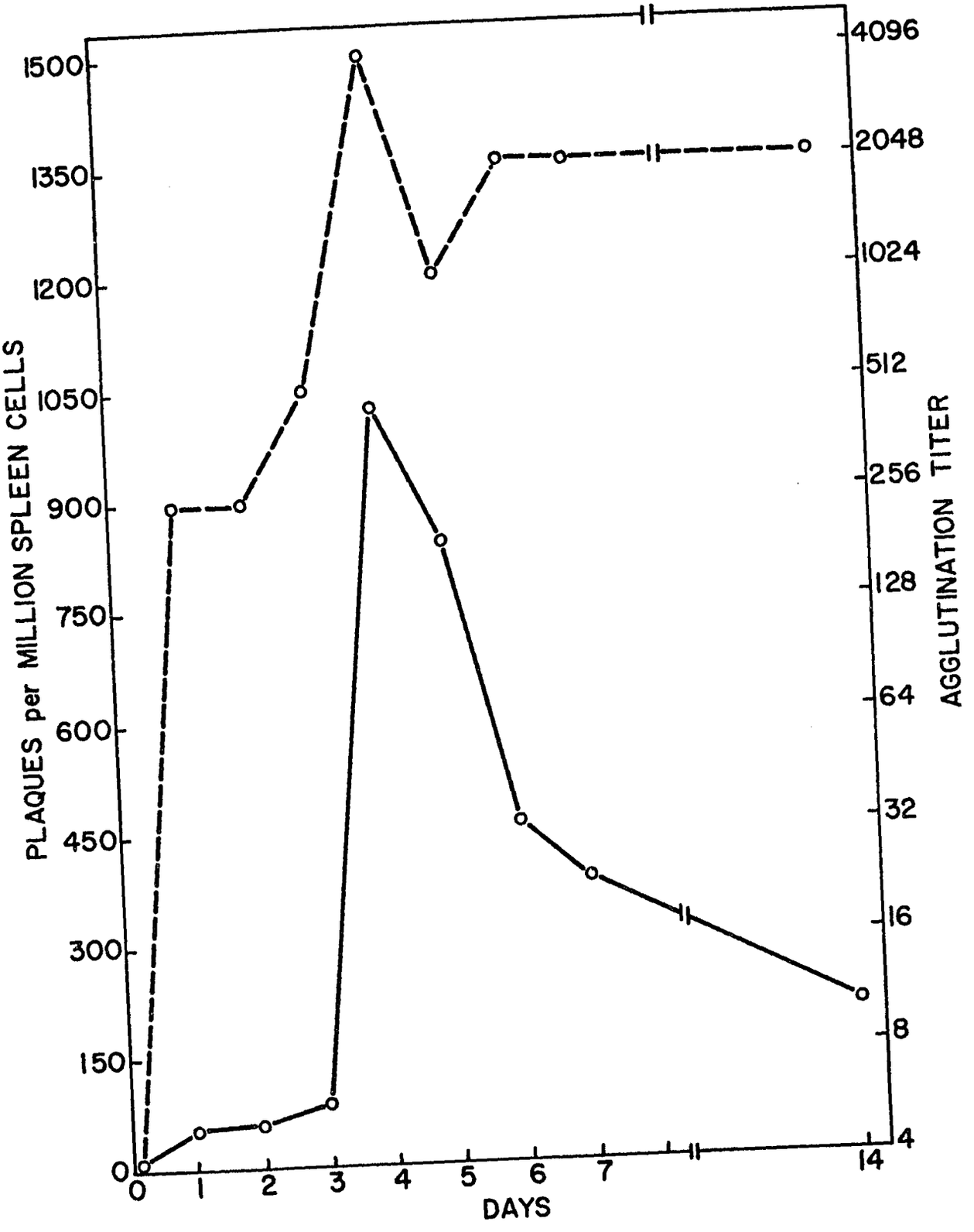
To characterize the response to a second injection of endotoxin, a previously stimulated group of mice was given a second injection of the antigen 14 days after the initial injection. The results are shown in Figure 2. An unexpected finding here was that when the serum titer of these animals was determined, it had dropped to 16. A loss of activity through serum processing can not be excluded here, since the animals showed a serum titer of 256 when tested on day 1 following the second stimulus. Plaque count was compatible with the earlier observations, showing approximately 60 plaque-forming cells on days 2 and 3, then rising sharply to a peak of 1024 on day 4. Again, excluding the results for day 0, the rise in agglutinin titer paralleled the rise in the number of competent cells rather closely. Although the peak response was reached one day earlier after the second injection, considerable variation was noted in these animals with respect to the time when maximum titers developed, and this finding probably reflects only differences in the physiological state of the mice at the time of antigenic challenge.

It is difficult to explain why the cellular response during the second injection was not as great as that noted after the primary stimulus. In this respect, secondarily stimulated cells may be capable of more active antibody synthesis than primarily

Figure 2. Plaque-forming cells and antibody response of mice following a second injection of 50 ug of *E. coli* 0127 endotoxin.

0——0 Plaque count

0----0 Agglutinin titer


PLAQUES per MILLION SPLEEN CELLS
1500
1350
1200
1050
900
750
600
450
300
150
0
AGGLUTINATION TITER
4096
2048
1024
512
256
128
64
32
16
8
4
0
1
2
3
4
5
6
7
14
DAYS

stimulated cells, or the contribution of the other antibody synthesizing tissues may be greater following a secondary stimulus. Another possibility is that cell types were present which were synthesizing antibody which was not detected by this method.

To test this latter possibility, the method of Sterzl et al. (1965) for the detection of cells of low hemolytic efficiency was employed. This consisted of flooding the test plate with an anti-mouse globulin serum and looking for an increase in plaque counts. When tested on day 7 following the second antigenic stimulation this method gave approximately a two-fold increase in the plaque count. While the increase would not be expected to be of this magnitude during the earlier phases of antibody production it would probably be sufficient to account for the discrepancy between the competent cell counts on the days of maximum titer. This finding may also have a bearing on the persistence of the high titers in the secondary response, since this method detects IgG antibody which is known to decay at a slower rate than the IgM or 19S globulins.

These data correlate with the data obtained from the study of the humoral response.

One possible objection to using the endotoxin of E. coli 0127 in studies of this nature is that this organism contains a "K" antigen and since the purity of the preparation is hard to assess, antibody directed against this antigenic constitutient could conceivably contribute to or be directly responsible for the plaques observed, rather than antibody specific for the endotoxin. In the studies presented here, the spleen cells were plated in the presence of cells which

were selected as "K" negative by the method of Edwards and Ewing (1962). No significant plaque count differences were noted between these plates and those plated with the routinely used culture.

The next experiments were designed to compare the response elicited by endotoxins to that obtained with intact bacterial cells. For this purpose an aqueous phenol extract of E. coli 0127 and formalinized whole cells prepared by the Rocky Mountain Laboratory were used. The dose level employed was 50 ug for both the toxin and the dried intact bacteria.

The results of both primary and secondary stimulation are shown in Table 10. There appeared to be no significant differences between the rate of appearance and decline of titers to these antigens. Although a greater agglutinin response was noted with the intact organisms, a maximum response was attained somewhat earlier with the toxin than with the bacteria. This difference is probably a reflection of the physical state of the antigen, as particulate antigens are usually better immunizing agents than soluble antigens. However, a second injection of the bacteria did not result in enhanced titers although the level of antibody remained higher for a longer period of time following a second stimulus. With the endotoxin it should be noted that titers rose above those of the primary response and remained at an elevated level throughout the experiment. Again the peak response to the toxin appeared earlier than that to whole cells. The significance of this finding is not readily apparent. Since there is obviously more endotoxin present in the purified preparation than the whole cells, and since

Table 10. Comparison of the immune response of mice to whole cells and endotoxin from E. coli 0127.

Antigen	Dose		Antibody Titer					
	1st inj.	2nd inj.	Days after antigenic challenge					
			0	1	3	5	7	14
Intact E. coli 0127	50 ug	0	8	8	256	2048	4096	1024
	50 ug	50 ug	8	512	512	1024	2048	2048
E. coli 0127 endotoxin	50 ug	0	8	16	8	1024	1024	256
	50 ug	50 ug	8	16	256	2048	2048	2048

endotoxin is capable of bringing about marked cellular proliferation and demonstrates potent adjuvant effects, the earlier peak titer may be a quantitative difference in the amount of endotoxin present.

A report by Rowley et al. (1964) demonstrated that the immune response to sheep erythrocytes could be blocked by the injection of specific antiserum. Since the response to endotoxins is similar to that described for red cells an attempt was made to suppress the immune response to endotoxins by the passive transfer of immune serum. The results of an attempt to block the response to primary stimulation with E. coli 0127 endotoxin is shown in Table 11. The passive antibody titer at time of antigenic challenge was 160. It can be seen from these results that attempts to block the immune response to this endotoxin were unsuccessful. Although Rowley et al. (1964) were able to show immune suppression of a primary stimulus with red cell antigens, their results could not be duplicated in the endotoxin system used in these studies.

Attempts to block the secondary response to sheep erythrocytes have not been successful as reported by Rowley et al. (1964). Attempts to block the response to a second injection of endotoxin using both E. coli 0127 and S. enteritidis (Se Pool 1) endotoxins were likewise unsuccessful. Therefore, while certain similarities do exist between the immune response to endotoxins and the immune response to sheep erythrocytes, these data indicate that the control mechanism for antibody formation to endotoxin is of a different nature than that proposed by Rowley et al. (1964) for red cell antigens on the basis of their immune suppression studies.

Table 11. The effect of passively transferred antibody on the immune response of mice to E. coli 0127 endotoxin.

Antibody Administered	Antigenic Challenge (24 hrs. after antibody injection)	Antibody Titer		
		Days after immunization		
		5	7	14
0.3 ml 0127 antiserum	50 ug 0127 toxin i.v.	640	2560	640
03 ml 0127 antiserum	none	40	40	0
0.3 ml normal mouse serum	50 ug 0127 toxin i.v.	320	1280	320
0.3 ml srbc antiserum	50 ug 0127 toxin i.v.	320	1280	320
none	50 ug 0127 toxin i.v.	640	2560	640

A final aspect of the characterization of the immune response of mice to the lipopolysaccharide antigens was concerned with determination of the molecular nature of the antibody response. While no detailed studies of this nature has been reported for mice, rabbits respond to endotoxins with the production of IgM antibodies. Rink (1965), however, obtained data which suggested that CD-1 mice made significant amounts of IgG antibody against E. coli 0127 endotoxin. It was of interest to determine if a similar response could be elicited in the RML mice.

Serum from mice immunized 5 days after a primary injection and 4 and 21 days after a secondary stimulation was subjected to sucrose density gradient ultracentrifugation. The titers of the unfractionated serum samples were 8192, 8192, and 4092, respectively. The results presented in Tables 12, 13, and 14 show the protein and antibody distribution in the various fractions obtained from these samples following centrifugation. In Table 12 it is apparent that at 5 days following a single injection of 50 ug of E. coli 0127 endotoxin, all the antibody is limited to the first protein peak, corresponding to the IgM or 19S region. No activity was found in the 7S region of the gradient. This is in agreement with most observations on the molecular nature of the immunoglobulins formed in response to endotoxins. However, as shown in Table 13, four days following a second injection of the antigen a significant amount of the antibody formed is of the IgG type. However, the major activity still lies within the 19S region. Twenty-one days following a second antigenic does, (Table 14), the titers in both regions are lower, but a seemingly

Table 12. Distribution of agglutinating activity and protein obtained by sucrose density ultracentrifugation of serum from mice injected five days previously with 50 ug of E. coli 0127 endotoxin.

Fraction number	O. D. at 280 mu	Agglutinin titer
1	0.045	0
2	0.025	0
3	0.023	0
4	0.025	0
5	0.03	0
6	0.03	0
7	0.055	32
8	0.135	128
9	0.155	128
10	0.075	16
11	0.05	0
12	0.120	0
13	0.290	0
14	0.585	0
15	0.875	0
16	1.25	0
17	0.90	0
18	0.375	0
19	0.195	0
20	0.38	0
Whole serum		8192

Table 13. Distribution of agglutinating activity and protein obtained by sucrose density ultracentrifugation of serum from mice injected four days previously with a second injection of E. coli 0127 endotoxin.

Fraction number	O. D. at 280 mu	Agglutinin titer
1	0.033	0
2	0.025	0
3	0.020	0
4	0.025	0
5	0.023	0
6	0.025	16
7	0.055	128
8	0.155	256
9	0.190	512
10	0.085	64
11	0.075	8
12	0.210	8
13	0.490	32
14	0.800	32
15	1.100	16
16	1.100	4
17	0.585	0
18	0.210	0
19	0.460	0
20	0.340	0
Whole serum		8192

Table 14. Distribution of agglutinating activity and protein obtained by sucrose density ultracentrifugation of serum from mice injected twenty-one days previously with a second injection of E. coli 0127 endotoxin.

Fraction number	O. D. at 280 mu	Agglutinin titer
1	0.030	0
2	0.020	0
3	0.020	0
4	0.030	0
5	0.020	0
6	0.020	0
7	0.050	8
8	0.120	8
9	0.165	32
10	0.070	16
11	0.110	0
12	0.165	0
13	0.410	8
14	0.760	16
15	1.200	16
16	1.200	0
17	0.600	0
18	0.220	0
19	0.290	4
20	0.280	8
Whole serum		4096

higher proportion of the antibody titer can be attributed to the 7S globulins. These observations confirm the work of Rink (1965) and show that appreciable IgG antibody is synthesized by mice after a second injection of E. coli 0127 endotoxin.

Although these results definitely demonstrated the presence of IgG globulins, the endotoxin used was a crude preparation obtained commercially. Therefore, serum samples from RML mice immunized with a more purified preparation of S. enteritidis (Se Pool 1) were then assayed by the same technique. Since no antibody was made on the first exposure of mice to this antigen, samples were taken at various time intervals after the secondary stimulation.

Table 15 shows the antibody distribution to Se Pool 1 toxin three days after secondary injection of the antigen. The agglutinin response at this time for the unfractionated serum was 256. Note that all of the antibody activity is confined to the 19S region. Although the total antibody is less than that described above, the distribution pattern is similar to that seen with E. coli 0127 prior to a second stimulus. As seen from Table 16, the rapid rise in titer between days three and four is due to an increased synthesis of IgM antibody. The whole serum titer on day four was 4096. Hence, up to the time of peak response the antibody synthesized is IgM following immunization with Se Pool 1 endotoxin.

The results of serum taken twenty-one days after the second antigenic stimulation is shown in Table 17. Only small amounts of antibody are present in the 7S region. However, it should be noted that the level of activity seen here may not reflect a true value for

Table 15. Distribution of agglutinating activity and protein obtained by sucrose density ultracentrifugation of serum from mice injected three days previously with a second injection of Se Pool 1 endotoxin.

Fraction number	O. D. at 280 mu	Agglutinin titer
1	0.015	0
2	0.020	0
3	0.030	0
4	0.033	0
5	0.033	0
6	0.075	4
7	0.150	8
8	0.195	4
9	0.115	0
10	0.080	0
11	0.120	0
12	0.320	0
13	0.610	0
14	1.00	0
15	1.500	0
16	1.200	0
17	0.680	0
18	0.220	0
19	0.175	0
20	0.270	0
Whole serum		256

Table 16. Distribution of agglutinating activity and protein obtained by sucrose density ultracentrifugation of serum from mice injected four days previously with a second injection of 50 ug of Se Pool 1 endotoxin.

Fraction number	O. D. at 280 mu	Agglutinin titer
1	0.020	0
2	0.025	0
3	0.010	0
4	0.015	0
5	0.025	0
6	0.030	0
7	0.085	16
8	0.180	64
9	0.180	64
10	0.080	8
11	0.125	0
12	0.340	0
13	0.700	0
14	1.000	0
15	1.400	0
16	1.000	0
17	0.520	0
18	0.165	0
19	0.270	0
20	0.310	0
Whole serum		4096

Table 17. Distribution of agglutinating activity and protein obtained by sucrose density ultracentrifugation of serum from mice injected twenty-one days previously with a second injection of 50 ug of Se Pool 1 endotoxin.

Fraction number	O. D. at 280 mu	Agglutinin titer
1	0.020	0
2	0.020	0
3	0.020	0
4	0.020	0
5	0.020	0
6	0.020	0
7	0.075	8
8	0.200	32
9	0.210	32
10	0.100	4
11	0.160	0
12	0.430	0
13	0.780	4
14	1.400	4
15	1.600	0
16	1.200	0
17	0.480	0
18	0.150	0
19	1.22	0
20	0.180	0
Whole serum		2048

the amount of active globulin present. Since it is known that 19S globulins are extremely more efficient in most serological tests than 7S globulins, the low activity seen here may in reality represent rather large amounts of the immunoglobulin. It seems apparent though, that even though some IgG antibody is undoubtedly being synthesized, it is not produced in the quantities noted in the response to E. coli 0127 endotoxin.

Figures 3, 4, and 5 depict the antibody and protein distribution of CD-1 mice serum samples taken at various stages of the immune response following injection with 50 ug of Ec 12/13 original endotoxin. Figure 3 represents samples taken at day 8 after a primary injection. The titer of the whole serum was 2048. This is in contrast to the observations with RML mice which were incapable of responding to a single injection of this antigen. Also of significance is the presence of small amounts of 7S globulin in this early phase of antibody production, a fact not observed with the other preparations.

Serum samples collected from mice 8 days after the second antigenic stimulation showed the pattern displayed in Figure 4. The serum titer at this time had risen to 32,788. This rise in titer is accompanied by an increase in activity associated with both the 19S and 7S globulin fractions. By day 24, (Figure 5), there was an even further increase in titer of the whole serum to 65,576. This manifested itself as an additional increase in activity in both the 19S and 7S fractions.

Figure 3. Distribution of agglutinating activity and protein obtained by sucrose density ultracentrifugation of serum from CD-1 mice injected eight days previously with 50 ug of Ec 12/13 original endotoxin.

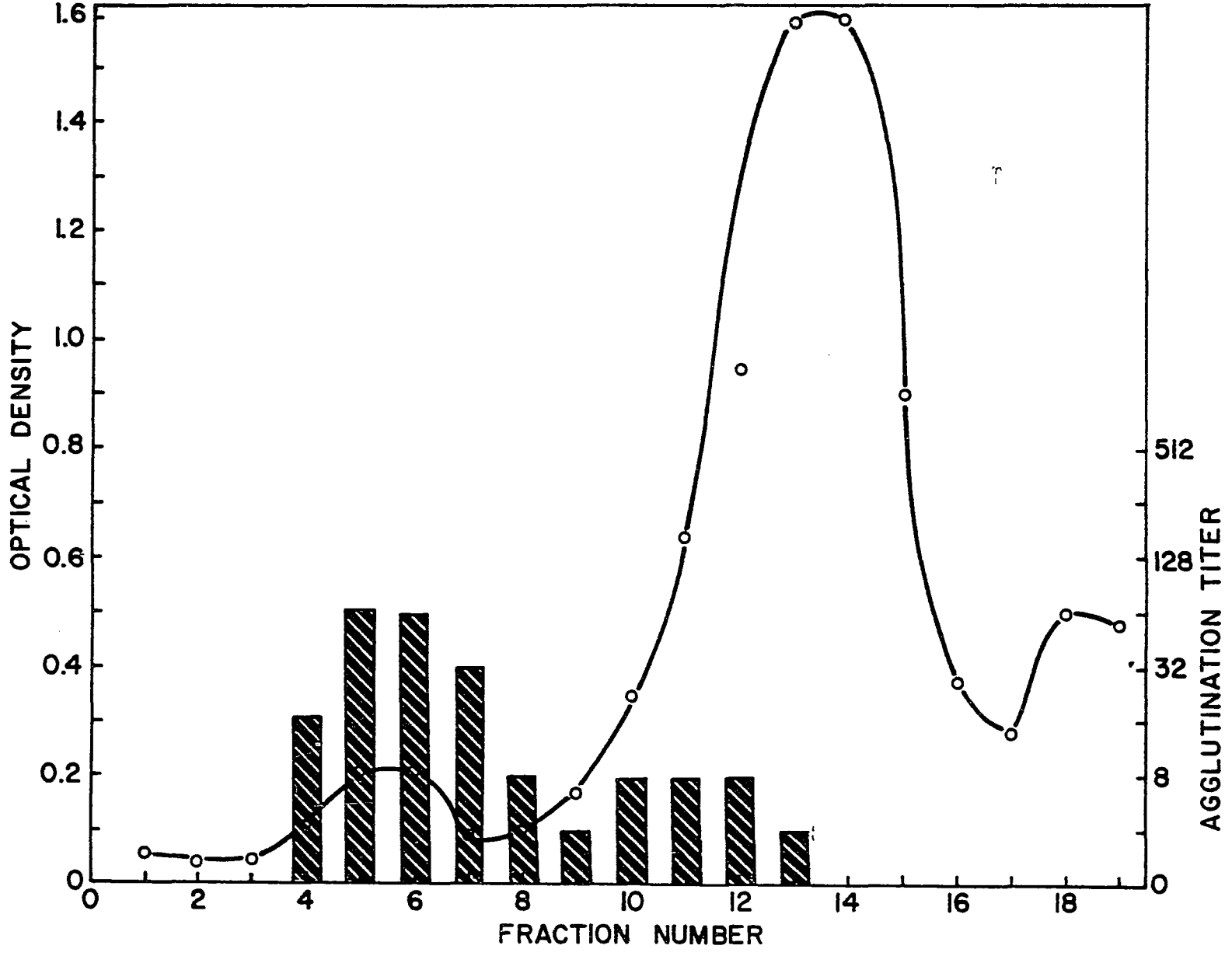

1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
OPTICAL DENSITY
0
2
4
6
8
10
12
14
16
18
FRACTION NUMBER
512
128
32
8
0
AGGLUTINATION TITER

Figure 4. Distribution of agglutinating activity and protein obtained by sucrose density ultra-centrifugation of serum from CD-1 mice injected eight days previously with a second injection of 50 ug of Ec 12/13 original endotoxin.

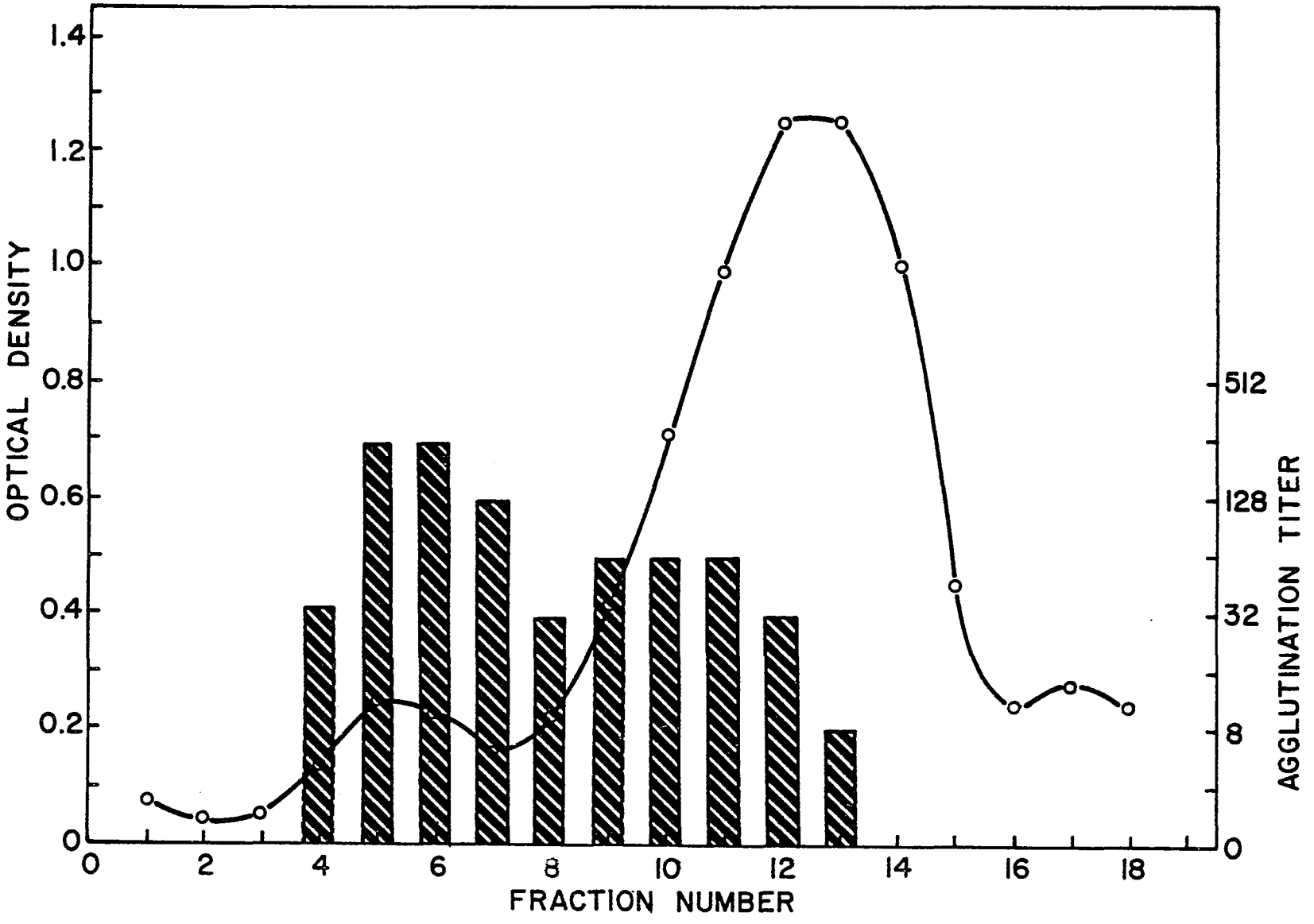
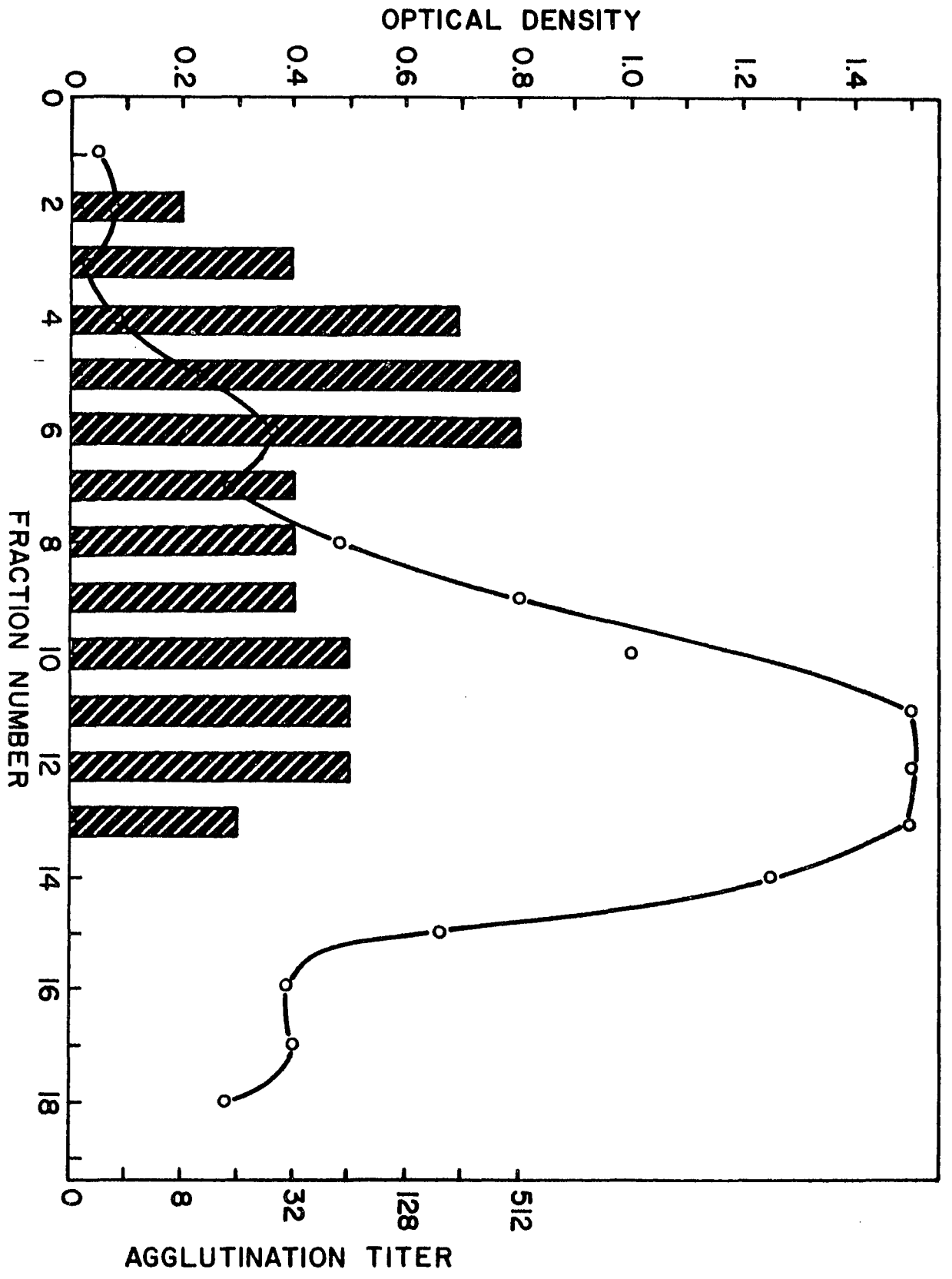

OPTICAL DENSITY
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
12
14
16
18
FRACTION NUMBER
512
128
32
8
0
AGGLUTINATION TITER

Figure 5. Distribution of agglutinating activity and protein obtained by sucrose density ultracentrifugation of serum from CD-1 mice injected twenty-four days previously with a second injection of 50 ug of Ec 12/13 original endotoxin.


OPTICAL DENSITY
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
FRACTION NUMBER
0
2
4
6
8
10
12
14
16
18
AGGLUTINATION TITER
0
8
32
128
512

The magnitude of these serum titers is quite surprising in view of the generally accepted concept of inability of mice to respond to these antigens. However, from the studies presented here, it is readily apparent that mice are quite capable of responding with very high agglutinin titers to injections of endotoxins. Furthermore, contrary to the findings in rabbits, a significant amount of the synthesized antibody, especially to a second antigenic challenge, appears to be of the IgG globulin class.

It is quite possible that the phenomena observed in these experiments are restricted to certain strains of mice. It can be readily seen, for instance, that CD-1 mice are capable of responding to a much greater extent than the RML strain to selected endotoxins.

In order to substantiate the involvement of the 7S globulins in the response of the mouse to endotoxins, the globulin fractions were tested for their sensitivity to treatment with 2-mercaptoethanol. This compound is capable of selectively destroying the 19S globulins. The fractions from the samples described in Tables 12 through 17, and Figures 3 through 5 were used in this study. All fractions under each peak from a particular serum sample was pooled and treated with 2-mercaptoethanol. After treatment the amount of antibody activity remaining was designated as 7S antibody. The results are shown in Table 18.

These findings are in good agreement with the sucrose density gradient studies concerning the molecular nature of the antibodies in the various fractions. It should be noted that in no case did any titer develop in fractions obtained from the 19S peak after

Table 18. Sensitivity of whole serum and sucrose gradient fractions to reductive cleavage with 2-mercaptoethanol.

Antigen	Days after Immunization	Fractions	Antibody Titers	
			Before 2-ME	After 2-ME
E. coli 0127	5 days after first inj.	19S	32	0
		7S	0	0
		Whole serum	8192	16
	4 days after second inj.	19S	128	0
		7S	16	32
		Whole serum	8192	128
	21 days after second inj.	19S	32	0
		7S	8	8
		Whole serum	4096	128
Ec 12/13 original	8 days after first inj.	19S	32	0
		7S	4	4
		Whole serum	2048	32
	8 days after second inj.	19S	256	0
		7S	32	64
		Whole serum	8192	512
	24 days after second inj.	19S	64	0
		7S	32	32
		Whole serum	*	*
Se Pool 1	3 days after second inj.	19S	8	0
		7S	0	0
		Whole serum	256	4
	4 days after second inj.	19S	32	0
		7S	0	0
		Whole serum	4096	16
	21 days after second inj.	19S	32	0
		7S	4	4
		Whole serum	2048	32

*Limited serum did not permit these determinations

treatment with 2-mercaptoethanol. Likewise, titers of the 7S peaks were not destroyed by this treatment. In all cases treatment of the whole serum with 2-mercaptoethanol greatly reduced the titer. However, even in the samples where no activity was shown to be associated with fractions of the 7S peak, fractionation by 2-mercaptoethanol failed to remove all the activity from the whole serum. It is possible that even in these sera some IgG globulin is present, but after fractionation by gradient centrifugation, they are diluted to such an extent as to not be detected by agglutination tests. It is apparent from these results that significant amounts of IgG antibody are present during the latter stages of the immune response of mice to the lipopolysaccharide antigens of gram-negative bacteria.

SUMMARY

The ability of mice to respond to antigenic stimulation with the lipopolysaccharide antigens of gram-negative bacteria was shown to be influenced by a number of factors. The response obtained to either a primary or secondary stimulation was dependent upon the antigenic dose employed as well as the interval between injections.

While the strain of mice used for these studies was generally incapable of responding with antibody production to the first injection of endotoxin this primary antigenic stimulus served to condition, in some unknown manner, the immune mechanism of the animal so that it elicited a "recall" type response to subsequent injections of the antigen. Titers following second injections were much higher than those obtained at any dose level after the first injection, and much greater in magnitude than titers generally reported in the literature for mice immunized with endotoxins. Like the primary response, the response to the second injection of the antigen is dose dependent.

Due to the many similarities between the responses elicited by the first and second antigenic challenges, it was not possible to say definitely that true "immunological memory" was demonstrated in mice for these antigens. While the magnitude of the titers produced to a second injection indicated this, the similarities in the rise and decline of antibody titers argued strongly against it.

The cellular response to both primary and secondary stimulation was shown to parallel rather closely the humoral antibody response.

No striking differences were noted between the response to a first and second injection with the exception of the possible involvement of cells producing 7S antibody during the secondary stimulation response.

Whole bacterial cells of E. coli 0127 elicited the same general response as did the endotoxin extracted from them. Titers were higher to the intact cells, which was interpreted to be a reflection of the physical state of the antigen.

Probably the most significant observation in the characterization of this response was the demonstration of IgG antibody in the response of the mouse to these antigens. In other animals only IgM antibody has been considered to be involved.

Differences in the globulin type synthesized was found to be a characteristic of the individual preparations used. More IgG antibody was produced to Ec 12/13 original endotoxin than to either E. coli 0127 or S. enteritidis endotoxin. However, since a different mouse strain was used in the study of the Ec 12/13 original endotoxin response, a comparison is difficult.

Although significant amounts of IgG antibody were found late in the response to E. coli 0127, no antibodies of this type were found prior to the second injection in the case of the other endotoxin preparations. CD-1 mice showed low levels of 7S antibody in response to the first injection.

The data presented challenges the long accepted concept that mice are incapable of responding with significant antibody titers to injections with the lipopolysaccharide antigens of gram-negative bacteria.

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VITA

Jerry Lee Allen was born June 30, 1936 in Calvin, Louisiana, the son of Mr. and Mrs. Thomas Lee Allen. He was graduated from Calvin High School in May of 1954. He received the degree of Bachelor of Science from the University of Southwestern Louisiana in August of 1958. Following graduation he served in the United States Army from March of 1959 through February of 1961. In June of 1962 he enrolled in the Graduate School of Northwestern State College in the Department of Microbiology and received the degree of Master of Science in August of 1964. He enrolled in the Graduate School at Louisiana State University in September of 1964.

EXAMINATION AND THESIS REPORT

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Major Field: Microbiology

Title of Thesis: Some Aspects of the Immune Response of Mice to the Lipopolysaccharide Antigens of Gram-negative Bacteria.

Approved:

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

Date of Examination:

January 10, 1967